Immunity, Vol. 4, 113–121, February, 1996, Copyright ©1996 by Cell Press

# IL-4 Function Can Be Transferred to the IL-2 Receptor by Tyrosine Containing Sequences Found in the IL-4 Receptor α Chain

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## Summary

**IL-4 binds to a cell surface receptor complex that consists of the IL-4-binding protein (IL-4Rα) and the γ chain of the IL-2 receptor complex (γc). The receptors for IL-4 and IL-2 have several features in common; both use the γc as a receptor component, and both activate the Janus kinases JAK-1 and JAK-3. In spite of these similarities, IL-4 evokes specific responses, including the tyrosine phosphorylation of 4PS/IRS-2 and the induction of CD23. To determine whether sequences within the cytoplasmic domain of the IL-4Rα specify these IL-4-specific responses, we transplanted the insulin IL-4 receptor motif (I4R motif) of the huIL-4Rα to the cytoplasmic domain of a truncated IL-2Rβ. In addition, we transplanted a region that contains peptide sequences shown to block Stat6 binding to DNA. We analyzed the ability of cells expressing these IL-2R–IL-4R chimeric constructs to respond to IL-2. We found that IL-4 function could be transplanted to the IL-2 receptor by these regions and that proliferative and differentiative functions can be induced by different receptor sequences.**

## Introduction

Treatment of cells with interleukin-4 (IL-4) elicits many different biological responses, including an increase in cell proliferation and the transcription of a series of genes, particularly those involved in B cell differentiation (Paul, 1991). Some of these responses are unique to IL-4, while others are also elicited by different cytokines. These responses are mediated by the IL-4 receptor complex, which consists of a 140 kDa high affinity binding chain (IL-4Rα) (Idzerda et al., 1990; Galizzi et al., 1990) and the common γ chain (γc) (Russell, et al., 1993; Kondo, et al., 1993) that is shared by the receptors for IL-2, IL-7, IL-9, and IL-15 (Takeshita et al., 1992; Noguchi et al., 1993; Kondo et al., 1994; Russell et al., 1994; Giri et al., 1994). Both chains of the IL-4 receptor complex are members of the hematopoietin receptor superfamily (Cosman, 1993). Like other members of this family, the IL-4 receptor does not contain any consensus sequences encoding tyrosine or serine/threonine kinases. However, it has been shown that IL-4Rα associates with the Janus family kinase JAK-1 (Yin et al., 1994) and the γc associates with JAK-3 (Russell et al., 1994; Miyazaki et al., 1994). Occupancy of the IL-4 receptor induces tyrosine phosphorylation of the 170 kDa protein, 4PS, and of IL-4Rα (Wang et al., 1992; Izuhara and Harada, 1993). 4PS is related to insulin receptor substrate-1 (IRS-1), a substrate of the insulin receptor kinase (Wang et al., 1993a), and also serves as a substrate for the insulin and insulin-like growth factor-1 (IGF-1) receptors.

IRS-1 contains more than 20 potential tyrosine phosphorylation sites and 30 potential serine/threonine phosphorylation sites (White and Kahn, 1994). The cDNA encoding 4PS has recently been cloned from the myeloid cell type FDC-P2 (Sun et al., 1995). The sequence for 4PS is not highly homologous to IRS-1, but it contains tyrosine phosphorylation sites similar to IRS-1. The designation IRS-2 has been proposed. Tyrosine-phosphorylated sites within IRS-1 and 4PS/IRS-2 associate with high affinity to cellular proteins that contain SH2 domains, including the p85 subunit of PI-3-kinase (PI-3-K), growth factor receptor–bound protein 2 (GRB2), the SH2 and SH3 domain containing adaptor protein nck, and the src-homology protein tyrosine phosphatase 2 (White and Kahn, 1994).

Using the myeloid progenitor cell line 32D-IRS-1 that expresses the cDNA for rat (r)IRS-1 (Wang et al., 1993b), we identified a region of human (hu)IL-4Rα, between amino acids 437 and 557 that is important for IL-4-induced cell growth and IRS-1 phosphorylation (Keegan et al., 1994a, 1994b). This interval contains a single Y residue surrounded by a sequence motif (491PLXXXXNPXYXSXSD505) that is homologous to sequences found in the insulin and IGF-1 receptors. This motif has been designated the insulin–IL-4 receptor motif (I4R motif). Mutation of the central Y residue of the I4R motif to an F in the huIL-4Rα (Keegan et al., 1994a) or the human insulin receptor (White et al., 1988) impairs the ability of these receptors to signal IRS-1 phosphorylation or to induce a proliferative response upon occupancy by their cognate ligand, demonstrating that the Y residue in the I4R motif is important for linking to the IRS-1 pathway in both the IL-4 and insulin receptor systems. The I4R motif has been proposed to be an IRS-1 docking site. Indeed, a direct interaction between IRS-1 and the I4R motif of the insulin receptor (O'Neill et al., 1994) and the huIL-4Rα (K. Nelms and W. E. P., unpublished data) has been demonstrated using the yeast two-hybrid system.

Information on the signaling mechanisms that the IL-4 receptor may use for the activation of gene expression has been obtained. Engagement of cytokine receptors can lead directly to the rapid tyrosine phosphorylation and nuclear translocation of factors involved in gene transcription, termed signal transducers and activators of transcription (STATs) (Darnell et al., 1994). Recent work has demonstrated that a factor related to the known STATs may be involved in the signaling pathway from the IL-4 receptor complex to the nucleus. Three groups initially identified a factor that is tyrosine phosphorylated by IL-4 and that binds to consensus sequences found within the promoter regions of IL-4-inducible genes (Kohler and Rieber, 1993; Kotanides

and Reich, 1993; Schindler et al., 1994). The cDNA for an IL-4-inducible DNA-binding factor has been cloned, found to be related to the STAT family, and designated IL-4–STAT or Stat6 (Hou et al., 1994; Quelle et al., 1995). Interestingly, it was found that tyrosine-phosphorylated peptides derived from the sequence of a region of the huIL-4Rα cytoplasmic domain downstream of the I4R motif could inhibit the ability of Stat6 to bind DNA in vitro (Hou et al., 1994). Recent evidence suggests that complete activation of DNA binding by STATs may depend on a serine/threonine kinase (Zhang et al., 1995; Boulton et al., 1995; Wen et al., 1995).

Although the receptors for IL-4 and IL-2 have several features in common, including their use of the γc as a receptor component, and their activation of JAK-1 and JAK-3 (Witthuhn et al., 1994; Johnston et al., 1994), IL-4 evokes responses that IL-2 does not, such as the tyrosine phosphorylation of 4PS in murine cells (Morla et al., 1988), activation of Stat6 (Quelle et al., 1995; Lin et al., 1995), and induction of a set of genes (i.e., CD23, class II molecules, and the germline transcripts of the γ1 and ϵ immunoglobulin heavy chain genes). We propose that sequences within the cytoplasmic domain of the IL-4Rα determine the IL-4-specific responses and that the region containing such sequences can act as a domain capable of functioning in other settings.

Our previous studies in the 32D cell line have demonstrated that the I4R motif plays a crucial role in regulating proliferation, while the presumed Stat6 docking motifs are largely dispensible for proliferation (Keegan et al., 1994a; Quelle et al., 1995; Pernis et al., 1995). Stahl et al. (1995) has shown that a 5 aa Stat3-binding motif derived from the cytoplasmic domain of gp130 was sufficient to direct the tyrosine phosphorylation of Stat3 in the context of the erythropoietin receptor cytoplasmic domain. However, they did not address whether the Stat3-binding motif had any effect on the activation of specific genes by cyokines that utilize gp130 as a receptor component or on any gp130-dependent biological response. Herein, we examine the ability of sequences derived from the huIL-4Rα to dictate IL-4-specific biologic and biochemical responses in two different model systems. We transplanted tyrosine-containing sequences from the huIL-4Rα cytoplasmic domain, including the I4R motif and the presumed Stat6 docking motifs, to the cytoplasmic domain of a truncated human IL-2Rβ. We found that IL-4-specific biological function could indeed be transplanted to the IL-2 receptor by these regions and that proliferative and differentiative functions could be transplanted by different receptor sequences.

## Results

To determine whether IL-4-specific signals could be mediated by regions of the IL-4 receptor, we "transplanted" two domains of the huIL-4Rα to a truncation of the IL-2Rβ chain (Figure 1). These domains contain tyrosine residues shown to be important for eliciting either a proliferative response (amino aacids 439–555, containing the I4R motif) (Keegan et al., 1994a) or the induction of DNA-binding activity (amino acids 558–657, containing two peptides shown to bind to Stat6) (Hou et al., 1994). The IL-2Rβ truncation is similar to the H truncation initially described by Hatakeyama et al. (1989) and is competent to stimulate tyrosine phosphorylation (Taniguchi and Minami, 1993; data not shown) and to associate with JAK-1 (Miyazaki et al., 1994), but lacks the tyrosine residues needed to recruit Stat5 (Lin et al., 1995; Fujii et al., 1995). The chimeric constructs were transfected into 32D-IRS-1 cells or the B cell lymphoma, M12.4.1. All the lines demonstrated similar levels of IL-2Rβ expression when analyzed by FACS staining using anti-huIL-2Rβ (Figure 2).

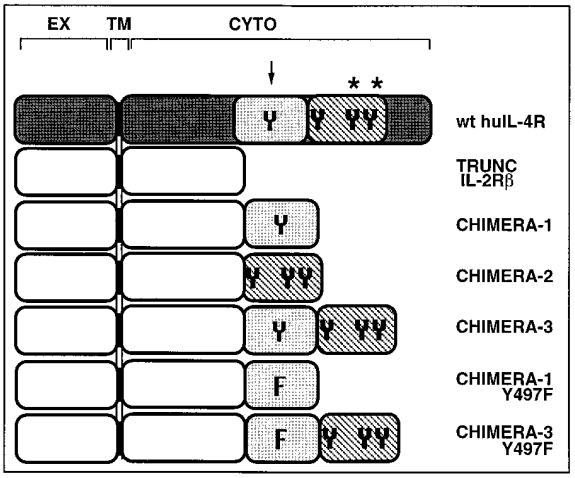


Figure 1. Map of Chimeric Receptors

The extracellular (EX), transmembrane (TM), and cytoplasmic domains of the huIL-4R (shaded) and truncated huIL-2Rβ (open) are shown. The Y residue within the I4R motif (amino acids 439–555, lightly shaded) is marked with the arrow, and the Y residues in the amino acid 558–657 domain (hatched) demonstrated to block a Stat6-mediated electrophoretic mobility shift are marked with asterisks. These domains were fused to a truncated huIL-2Rβ in wild-type and mutant form as indicated.

We analyzed the ability of the transfected 32D-IRS-1 lines to proliferate in response to IL-2 (Figure 3). In a 3 day proliferation assay, IL-2 caused little growth of cell lines expressing the IL-2Rβ construct truncated at amino acid 378 (TRUNC) (Figure 3A), a result similar to that reported by Goldsmith et al. (1994). Cell lines expressing chimeric receptors that include the domain containing the I4R motif alone (CHIM-1) proliferated in response to IL-2, while the chimeric receptor lacking the I4R-containing domain (CHIM-2) did not (Figure 3B). An unexpected finding was that cell lines expressing CHIM-1 showed greater tritiated thymidine uptake in response to various concentrations of IL-2 than did lines expressing CHIM-3 (Figures 3B and 3C). Since CHIM-3 is comprised of both the I4R-containing domain and the domain comprised of amino acids 558–657, this result suggests that the region between amino acids 558–657 contains a negative regulator of the function of the I4R motif or of cell growth. The importance of the Y in the I4R motif is shown by the finding that cell lines expressing chimeric receptors with Y to F mutations in the I4R motif (CHIM-1Y497F and CHIM-3Y497F ) showed marked impairment in IL-2-induced proliferation (Figure 3B). All the cell lines responded vigorously to IL-3 and they all responded to mIL-4 in a short term (24 hr) assay (Figure

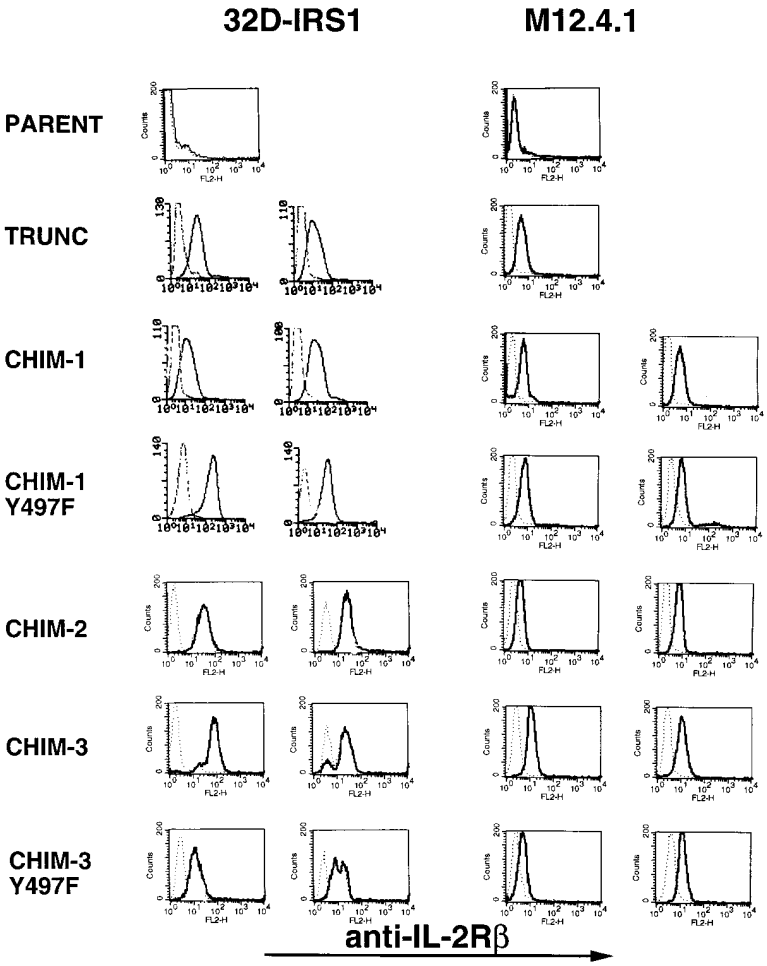


Figure 2. Levels of huIL-2Rβ Expression

Expression of the chimeric constructs was determined by staining with anti-huIL-2Rβ as described in Experimental Procedures. Cells were stained in the presence (solid line) or absence (dotted line) of the first antibody.

3D) as expected (Wang et al., 1993b; Keegan et al., 1994a).

We also analyzed the ability of the chimeras to stimulate the tyrosine phosphorylation of IRS-1 and its association with the SH2 domain–containing molecules PI-3-K and GRB2 (Figure 4). The 32D-IRS-1 lines demonstrated some constitutive tyrosine phosphorylation of IRS-1, as shown by direct anti-phosphotyrosine blots of cell lysates (Figure 4A) and of anti-IRS-1 immunoprecipitates of the parental 32D-IRS-1 cell line (Figure 4B) and by the constitutive association of IRS-1 with p85 (Figure 4C). However, we found that stimulation of wild-type CHIM-1- or CHIM-3-expressing lines with IL-2, but not TRUNC- or CHIM-2-expressing lines, induced the association of IRS-1 with GRB2 (Figure 4C). Since IRS-1 posseses only one consensus site for GRB2 binding (Y895VNI), and the interaction of IRS-1 with GRB2 is dependent on tyrosine phosphorylation (White and Kahn, 1994), these results suggest that the IRS-1 residue involved in GRB2 binding is not constitutively phosphorylated in these cells, but that its phosphorylation is stimulated by signaling through the I4R motif of the huIL-4Rα. CHIM-1 or CHIM-3 expressing the Y497F mutation were deficient at stimulating association of IRS-1 with GRB2 in response to IL-2 (densitometric analysis of band intensities for CHIM-1 or CHIM-1Y497F stimulated with nothing, IL-4, or IL-2 were as follows: 0.11, 2.0, 2.5; 0.1, 2.38, 0.3 arbitrary units and, for CHIM-3 or CHIM-3Y497F: 0.125, 2.18, 2.3; 0.15, 2.26, 0.13). Thus, the addition of IL-4R sequence containing the wild-type I4R motif confers the ability of the truncated IL-2Rβ to signal an enhanced proliferative response that is correlated with the association of IRS-1 with GRB2.

To determine the ability of huIL-4Rα sequences to confer differentiation functions, we analyzed the capacity of the chimeric IL-2 receptors to induce IL-4-specific responses in the B cell lymphoma, M12.4.1. The transfectant expressing TRUNC did not demonstrate any IL-2-induced tyrosine phosphorylation of 4PS as detected by precipitation with anti-phosphotyrosine or an anti-IRS-1 antisera capable of reacting with 4PS followed by anti-phosphotyrosine blotting (Figure 5). The ability to signal the tyrosine phosphorylation of 4PS correlated with the expression of a wild-type I4R motif (CHIM-1 and CHIM-3); chimeras with Y497F showed greatly diminished IL-2-induced phosphorylation of 4PS (densitometric analysis of band intensities of anti-IRS-1 precipitates for CHIM-1 or CHIM-1Y497F stimulated with nothing, IL-4, or IL-2 were as follows: 0.0, 14.8, 7.0; 0.0, 15.0, 0.0 arbitrary units and, for CHIM-3 or CHIM-3Y497F: 0.0,13.2, 9.4; 0.0, 10.7, 1.2). CHIM-2, lacking the I4R motif, had no activity in this assay. These results demonstrate that Y497 and its surrounding sequence can convey this IL-4 function to the IL-2 receptor.

We found that the capacity to induce the expression of CD23 could also be conferred on the IL-2 receptor

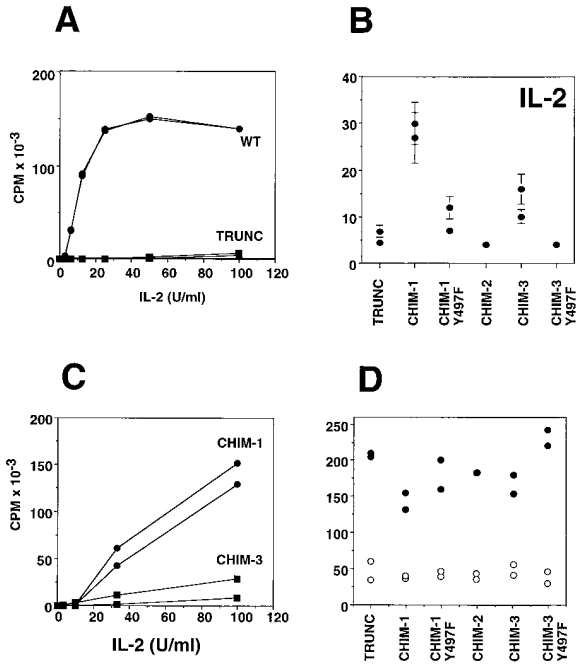


Figure 3. Proliferation of 32D-IRS-1 Cells in Response to IL-2

(A) 32D-IRS-1 cells expressing wild-type (circles) or truncated (squares) huIL-2Rβ were incubated for 72 hr in the presence of various amounts of IL-2. [$^3$H]thymidine was added during the last 5 hr of culture. Two clones are shown for each construct and each point represents the mean of triplicate wells.

(B) 32D-IRS-1 cells expressing the indicated constructs of huIL-2Rβ were incubated for 72 hr in the presence of 100 U/ml of IL-2. [$^3$H]thymidine was added during the last 5 hr of culture. Two clones are shown for each construct and each point represents the mean of triplicate wells plus or minus the error expressed as percentage of coordinate.

(C) As in (B), except that various concentrations of IL-2 were used to stimulate the clones expressing CHIM-1 (circles) and CHIM-3 (squares).

(D) As in (B), except the clones were treated with 5% Wehi-3-conditioned medium as a source of IL-3 for 72 hr (closed circles) or with 5 ng/ml mIL-4 for 24 hr (open symbols). [$^3$H]thymidine was added during the last 5 hr of culture.

by tyrosine containing sequences derived from the huIL-4Rα chain (Figure 6). Cell lines expressing chimeric receptors that include the defined Stat6 binding sites (CHIM-2 and CHIM-3) showed induction of CD23 by IL-2 that was only slightly less than that induced by mouse IL-4. Cell lines expressing CHIM-1, which lacks the defined Stat6 binding sites, did show induction of CD23 in response to IL-2, but to a lesser degree than lines expressing CHIM-2 or CHIM-3. Y497, of the I4R-motif, appears to be important in this modest induction of CD23 by CHIM-1, since mutant CHIM-1 (Y497F) showed no induction of CD23 whatsoever. The ability of IL-2 to induce STF-IL-4 activity correlated with the CD23 induction assay (data not shown).

Despite the fact that CHIM-3-Y497F contains intact Stat6 binding sites, lines expressing this chimera show a marked reduction in CD23 induction in response to IL-2 when compared with lines expressing either CHIM-2 or wild-type CHIM-3. This result was obtained with a series of independent stable B lymphoma lines expressing transfected receptors. The degree of receptor expression by CHIM-3-Y497F lines was comparable to those expressed by the other stable transfectants. This indicates that the Y497F mutation results in a strong negative regulation of CD23 induction and confirms the concept of cross-domain regulation already noted by the greater degree of IL-2-induced proliferation of 32D-IRS-1 cells expressing CHIM-1 than those expressing CHIM-3.

## Discussion

One of the features of cytokine biology is the great amount of pleiotropy and redundancy that exists among the cytokines. This occurs both at the functional level and at the biochemical level. The hematopoietin receptors that utilize the γc as part of their receptor complex (IL-2, IL-4, IL-7, IL-9, IL-15) share receptor subunits and basic signaling elements, such as the activation of Janus family tyrosine kinases and STAT family members. Yet each of these cytokines has the capacity to elicit a very specific set of biologic responses. Therefore, a major question is how do these different receptors, using many common elements, signal specific sets of events. The concept has now emerged that specific sequences in the cytoplasmic domains of receptors recruit unique sets of signaling molecules to the activated receptor complex (reviewed by Ivashkiv, 1995). In this study, we have examined the ability of sequences derived from the huIL-4Rα to dictate IL-4-specific biologic and biochemical responses in two different model systems.

We analyzed the ability of huIL-4Rα sequences, in the context of the IL-2Rβ that shares a receptor subunit and activates the same Janus kinases as the IL-4 receptor, to direct a growth response in the 32D-IRS-1 cell system. 32D cells express endogenous IL-4 receptors, although they do not proliferate in response to IL-4 and IL-4 does not induce detectable tyrosine phosphorylation of 4PS (Wang et al., 1993b). However, expression of the cDNA encoding rIRS-1 in these cells (32D-IRS-1 cells) increases their mitogenic response to IL-4. In these transfected cells, IL-4 readily induces the tyrosine phosphorylation of IRS-1.

In the 32D-IRS-1 cells expressing chimeric receptor constructs, the ability of IL-2 to initiate proliferation correlates with expression of the domain possessing a wild-type I4R motif and the association of IRS-1 with GRB-2. The SH2 domain of GRB2 binds to a sequence of IRS-1 (YVNI) containing Y895 (Skolnik et al., 1993). Mutation of Y895 to F (Y895F) impairs the ability of GRB-2 to associate with IRS-1 in vitro and in vivo. It has been shown that 32D cells expressing IRS-1Y895F are still able to proliferate in response to insulin (Myers et al., 1994a), suggesting that the formation of IRS-1–GRB2 complexes is not essential for insulin-stimulated mitogenesis. However, insulin treatment also activates the formation of shc–GRB2 complexes and activates the mitogen-activated protein (MAP) kinases and this pathway may be responsible for insulin-stimulated mitogenesis (Pruett et al., 1995). It has been reported that IL-4

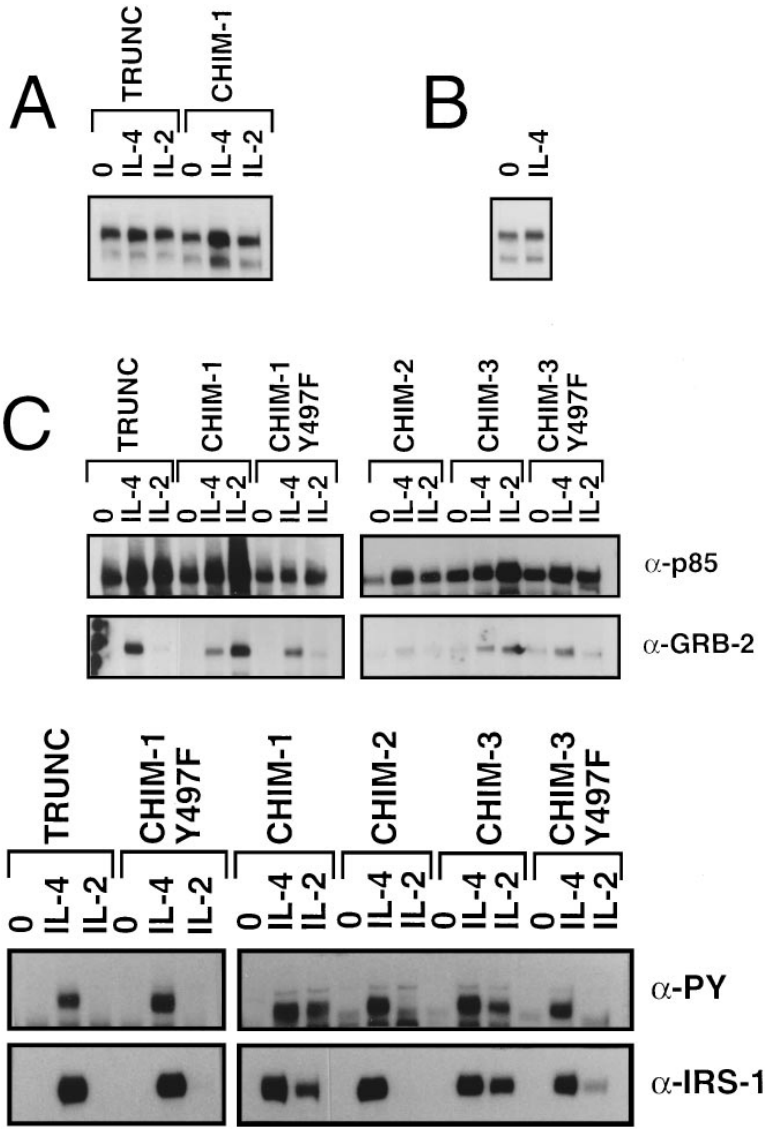


Figure 4. Ability of IL-4R–IL-2R Chimeras to Induce Association of IRS-1 with GRB2

Lysates were prepared from the indicated cells and separated by SDS–PAGE directly (A) or after precipitation with anti-IRS-1 (B). These Western blots were probed with anti-phosphotyrosine. (C) 32D-IRS-1 cells expressing the indicated IL-2Rβ constructs were treated with nothing, IL-4 (5 ng/ml), or IL-2 (100 U/ml). Lysates from treated cells were prepared and precipitated with anti-p85 or anti-GRB2 as indicated. Western blots of SDS gels were probed with anti-phosphotyrosine. The region of the blot containing IRS-1 is shown.

Figure 5. Ability of Chimeric Receptors to Signal 4PS Phosphorylation in Response to IL-2

M12.4.1 cells expressing the indicated constructs of IL-2Rβ were treated with nothing, IL-4 (5 ng/ml), or IL-2 (100 U/ml). 4PS phosphorylation was detected by immunoprecipitation with anti-phosphotyrosine or anti-IRS-1 followed by Western blotting with anti-phosphotyrosine. The region of the blot containing 4PS is shown.

does not strongly activate these pathways (Satoh et al., 1991; Welham et al., 1992) and, therefore, the mitogenic response to IL-4 may be more dependent on the formation of IRS-1–GRB2 complexes. We cannot rule out the importance of activation of PI-3-K in the mitogenic response to IL-4. We observed a constitutive level of tyrosine phosphorylation of IRS-1 and a constitutive association of IRS-1 with the p85 subunit of PI-3-K. However, there are 9 potential and 3 known sites of interaction for PI-3-K (YXXM) in the IRS-1 molecule (White and Kahn, 1994) and it is possible that the activity of this enzyme is increased only after binding to specific sites in the IRS-1 molecule (Myers et al., 1994b). Although the biochemical basis of IL-4-induced growth is not completely clear, the activation of the IRS-1 or 4PS pathway can be induced by transferring the domain containing the wild-type I4R motif, confirming our earlier work. This is especially clear in the transfected M12.4.1 cells that express physiologic levels of 4PS.

Interestingly, IL-2 caused a consistently greater proliferative response in cell lines expressing CHIM-1 than in those expressing CHIM-3. We had made similar observations in our earlier studies on deletion constructs of the huIL-4Rα (Keegan et al., 1994a). On average, 32D-IRS-1 cells expressing the huIL-4Rα truncated at amino acid 657 proliferated less well than those cells expressing a receptor truncated at amino acid 557. These results suggest that the sequences in the amino acid 558–657 domain may down-regulate the proliferative pathway activated by the I4R motif.

IL-4-specific differentiation function could also be transplanted to the IL-2 receptor by Y-containing sequences. Either the domain containing the I4R motif or the domain containing the defined Stat6 binding site conveys to the IL-2R the ability to induce CD23, although to differing degrees. Surprisingly, when the two domains were expressed together, we observed that expression of Y at position 497 of the I4R motif played a critical role in gene induction, suggesting some complex interaction between the two domains or between the signaling pathways they activate. The nature of the interaction is currently unknown but could potentially be linked to serine/

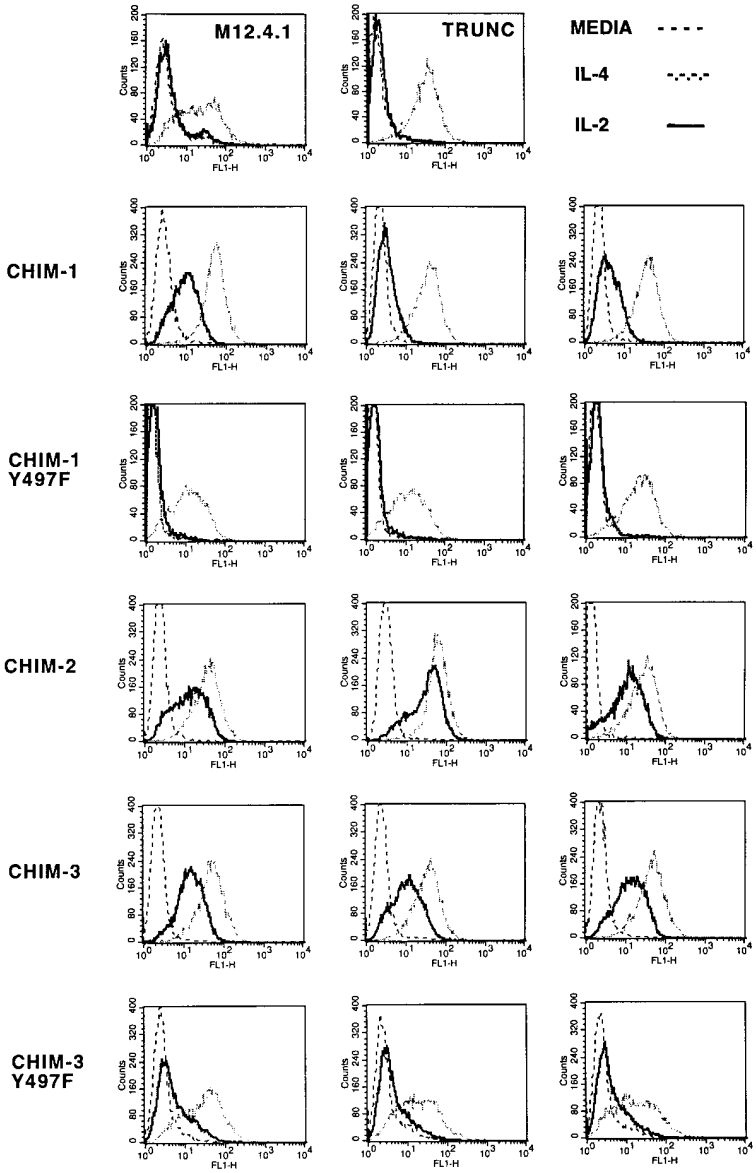


Figure 6. CD23 Induction in Response to IL-2

M12.4.1 cells expressing the indicated constructs of IL-2Rβ were treated with media (dashed line), mouse IL-4 (dotted line), or human IL-2 (solid line) for 48 hr. CD23 induction was analyzed by FACS using FITC–anti-CD23. Three individual clones expressing the various chimeric receptors are shown.

threonine kinase activation (Zhang et al., 1995; Boulton et al., 1995; Wen et al., 1995) or perhaps to an increased activation of a tyrosine phosphatase. Alternatively, it may reflect a domain–domain interaction, which may prevent access of substrates. Phosphorylation of key tyrosine residues in one or both domains upon receptor ligation may disrupt the interaction and allow efficient docking of substrate.

Although Y497 of the huIL-4Rα is important for linking to both the 4PS/IRS-1 pathway and the gene induction pathway, recent data suggests that the DNA binding activity of Stat6, as detected by gel shift assay, is not dependent on 4PS expression in the 32D cell system (Pernis et al., 1995). Recently, it was shown that two phosphopeptides derived from the STAT domain of the huIL-4R could block the binding of Stat6 to DNA (Hou et al., 1994), suggesting that these residues were responsible for recruiting Stat6 to the activated IL-4 receptor complex; in this assay, phosphopeptides containing Y497 failed to block binding to DNA. In addition, we did not observe the tyrosine phosphorylation of Stat6 in response to huIL-4 in 32D cells expressing the d557 truncation mutant, a construct expressing Y497 (Quelle et al., 1995). However, we have observed IL-4-induced gel shift activity in the d557-expressing 32D cells (Pernis et al., 1995), suggesting that the gel shift assay is more sensitive than the Western blotting assay.

In this study, we have clearly shown that the domain including the I4R motif can direct CD23 induction (and gel shift activity, data not shown) in transfected M12.4.1 cells and that expression of Y497 is required for this effect. Ryan et al. (1996 [this issue of *Immunity*]) found that CD23 induction in M12.4.1 is tightly linked to EMSA activity containing Stat6. In addition, a region of the murine CD23 promoter containing a putative Stat6 binding site (Kohler and Rieber, 1993; Kotanides and Reich, 1993) was shown to regulate the IL-4-induced transcription of a reporter construct (Richards and Katz, 1994).

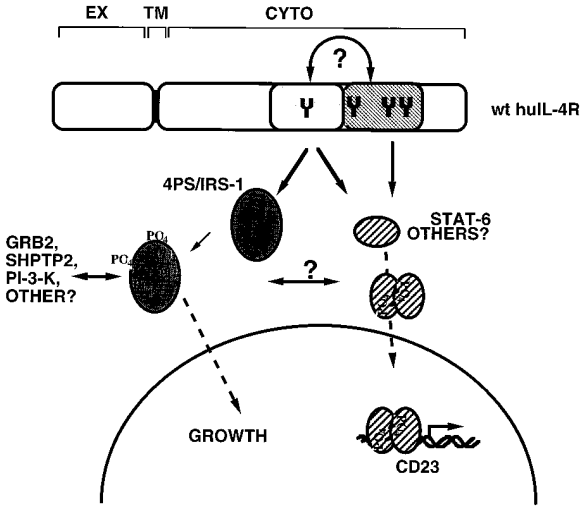


Figure 7. Y-Containing Sequences within the Cytoplasmic Domain of the IL-4Rα Convey IL-4-Specific Responses

A diagram of the signaling pathways activated by transplanted regions of the huIL-4Rα is shown. The domain containing the I4R motif (amino acids 439–555) is lightly shaded. The domain containing peptide sequence shown to interact with Stat6 (amino acids 558–657) is hatched. The domain containing the I4R motif can activate both the 4PS/IRS-1 pathway and the gene induction pathway. The amino acids 558–657 domain strongly activates the gene induction pathways, but does not affect the 4PS/IRS-1 pathway. The cross-regulation of signaling function by the two domains at the receptor level or at the signaling level is indicated by the question marks.

Therefore, it is likely that the I4R motif can direct the activation of Stat6 or a protein that is immunologically related to the cloned murine Stat6. It is possible that Stat6 has different affinities for the different tyrosine residues of the IL-4R; the interaction of Stat6 with the peptide containing Y497 may not have sufficient binding affinity to block the DNA binding activity in an in vitro assay. Indeed, the sequence surrounding Y497 is AYRSF, which has some similarity to the prototypic Stat6 binding site GYKXF.

Our results support the current hypothesis that specific tyrosine motifs in the cytoplasmic domains of cytokine receptors, when phosphorylated, physically interact with the SH2 domains of STATs. Several groups have found that phosphopeptides derived from receptor sequences block DNA binding activity of the activated STAT (Greenlund et al., 1994, 1995; Hou et al., 1994; Lin et al., 1995). In addition, it has been shown that the activation of a particular STAT by a cytokine is determined by the specific SH2 domains present in the STAT (Heim et al., 1995) and the presence of the appropriate Y-containing binding site in the cytokine receptor (Greenlund et al., 1995; Schindler et al., 1995). Indeed, Stahl et al. (1995) found that the sequence motif YXXQ, derived from the cytoplasmic domain of gp130, was sufficient to direct the tyrosine phosphorylation of Stat3 in the context of the erythropoietin receptor.

Here, we have shown that Y-based sequence motifs found in distinct cytoplasmic domains of the IL-4-Rα differentially activate biochemical pathways leading to biological outcomes, cell growth or gene expression (Figure 7). These domains function efficiently in the context of the IL-2 receptor, which shares certain homologies with the IL-4 receptor, notably an association with JAK-1 and γc. It remains to be determined whether these sequence motifs will also direct IL-4-function in another receptor background that is structurally divergent from the IL-4 receptor and is not, in particular, associated with members of the Janus kinase family.

## Experimental Procedures

### Cells and Reagents

The IL-3-dependent myeloid cell line expressing the cDNA encoding rat IRS-1 (Wang et al., 1993b), 32D-IRS-1 (obtained from Dr. J. H. Pierce, National Cancer Institute), was maintained in RPMI supplemented with glutamine, penicillin–streptomycin, 10% fetal calf serum (C-RPMI), and 10% WEHI-3 conditioned medium. The B cell lymphoma M12.4.1 (obtained from Dr. R. Asofsky, National Institutes of Health) was maintained in C-RPMI in the absence of cytokines. Recombinant muIL-4 expressed in baculovirus was affinity purified as described (Ohara et al., 1987). Recombinant huIL-2 was a gift from Dr. S. Rosenberg (National Cancer Institute).

### Construction of Chimeric Receptors

The IL-4R–IL-2R chimeras were generated using a two-step overlapping polymerase chain reaction (PCR) strategy (Higuchi, 1990). For example, details for the construction of chimera-1 are given. Details for the other constructs are available on request. Sense oligonucleotide (βchim-1) corresponding to the sequence just 5′ to the AatII site of the human IL-2Rβ (960 bp) and anti-sense oligonucleotide (βchim-2) containing sequence derived from the huIL-4R 5′ to the I4R motif (1490 bp) and sequence derived from the IL-2Rβ (1343 bp), were used to amplify a 407 bp fragment from the huIL-2Rβ–pME18s template plasmid (obtained from Dr. W. Leonard, National Heart, Lung, and Blood Institute). We used pfu polymerase (Stratagene, La Jolla, California) to reduce PCR-generated mutations. Sense oligonucleotide complementary to βchim-2 (βchim-3) and an anti-sense oligonucleotide derived from huIL-4R sequence 3′ to the I4R motif (1840 bp) and containing translational stop and XbaI sites (βchim-4) were used to amplify a 360 bp fragment from the huIL-4R–LTR-2 template plasmid containing cDNA for either wild-type or Y497FhuIL-4R. The two PCR products were gel purified, mixed in equal proportions, and then used as template for a second PCR reaction with βchim-1 and βchim-4 as primers. The 767 bp fragment was purified and digested with AatII and XbaI and ligated into the IL-2Rβ–pME18s vector cut with AatII and XbaI. Sequence was verified by the Sanger dideoxy chain–termination method and analyzed on an automated sequencing machine. The amino acids contained within the chimeras are as follows: TRUNC, 2R(1–378); CHIM-1, 2R(1–378) 4R(439–555); CHIM-2, 2R(1–378) 4R(558–657); CHIM-3, 2R(1–378) 4R(439–657). Amino acid numbering begins +1 with the signal peptide for huIL-4R and with the amino acid after the signal peptide for huIL-2Rβ There are 25 aa in the signal peptide of the huIL-4R and 26 for huIL-2Rβ.

### Transfection and Screening of Chimeric Constructs

32D-IRS-1 and M12.4.1 cells were washed two times in phosphate-buffered saline (PBS) and resuspended in PBS at $6.7 \times 10^7$/ml. For each transfection, $2 \times 10^7$ cells were used, mixed with 10 μg of chimeric DNA and 1 μg of vector carrying neomycin resistance, and subjected to electroporation using a Bio-Rad gene-pulsar set on 200 V and 960 μFa. After transfection, the cells were cultured overnight in the appropriate media before selection with 800 μg/ml G418 (Life Technologies, Grand Island, New York). Neomycin-resistant wells were tested for expression of IL-2Rβ by FACS analysis using biotin–anti-human IL-2Rβ (Endogen, Boston, Massachusetts) followed by strep–avidin–phycoerythrin (Southern Biotechnology, Birmingham, Alabama). The anti-Fc receptor antibody 2.4G2 was used to block Fc binding.

### Cellular Proliferation Assays

For the study of cellular proliferation, the cells were incubated at 5000 cells per well in a final volume of 0.2 ml of complete RPMI in the presence or absence of muIL-3 or huIL-2 for 72 hr at 37°C. The wells were pulsed with 1 μCi/well [$^3$H]thymidine for the final 4 hr of culture before harvesting using a Packard harvestor and the Matrix 9600 direct β count system.

### CD23 Induction Assay

M12.4.1 cells expressing the various constructs of the IL-2Rβ were incubated at $1 \times 10^5$/ml in C-RPMI in the presence or absence of muIL-4 (5 ng/ml) or huIL-2 (100 U/ml) for 48 hr at 37°C. Expression of murine CD23 was tested by FACS analysis using fluorescein isothiocyanate (FITC)–B3B4 (anti-murine CD23), a gift from Dr. D. H. Conrad (Virginia Commonwealth University), in the presence of the anti-Fc receptor antibody 2.4G2 to block Fc binding, before analysis on a FACScan (Becton-Dickinson).

### Immunoprecipitation and Immunoblotting

Analysis of phosphotyrosine containing proteins was performed as previously described (Keegan et al., 1994a). Cells were preincubated in RPMI without serum or cytokines for 2 hr at 37°C. After washing, the cells were resuspended in RPMI with 50 μM $Na_3VO_4$ and incubated in the presence or absence of muIL-4 (5 ng/ml) or huIL-2 (100 U/ml) for 10 min at room temperature. The reaction was terminated by 10-fold dilution in ice-cold PBS containing 100 μM $Na_3VO_4$. Cell pellets were lysed in HEPES lysis buffer (50 mM HEPES, 150 mM NaCl, 0.5% NP-40, 1 mM $Na_3VO_4$, NaF, pyrophosphate, 1 mM PMSF, and protease inhibitor cocktail) and clarified. The soluble fraction was immunoprecipitated with a monoclonal anti-phosphotyrosine antibody, 4G10, polyclonal anti-p85, or anti-GRB2 (all obtained from UBI, Lake Placid, New York) or rabbit anti-IRS-1 (a gift from Drs. L. M. Wang and J. H. Pierce, National Cancer Institute). The precipitates were washed three times in lysis buffer and solubilized in SDS sample buffer. The samples were separated on a 7.5% SDS–polyacrylamide gel before transfer to PVDF membrane. The membranes were blocked with PBS, 3% bovine serum albumin, 0.05% Tween-20. The blots were probed with anti-phosphotyrosine (1 μg/ml). The bound antibody was detected using enhanced chemiluminescence (Amersham, Arlington, Heights, Illinois). Densitometry was performed using either the RFLP-scan software from Stratagene (Figure 4) or the NIH-Image software (Figure 5).

### Acknowledgments

We would like to acknowledge Drs. J. Pierce and L.-M. Wang for the parental 32D-IRS-1 cells, the rabbit anti-IRS-1, and for advice and discussions during the course of this work; Dr. W. Leonard for the IL-2Rβ plasmid and advice on the chimera construction; and Dr. D. H. Conrad for the FITC–anti-CD23. We would also like to acknowledge Drs. D. W. Scott, K. Nelms, J. Ryan, and J. Zamorano for advice and discussions during the course of this work. This work was supported by American Red Cross funds.

Received September 6, 1995; revised November 13, 1995.

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